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Mechanism of Transcriptional Regulation by Methyl-CpG Binding Protein MBD1

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MBD1 is a mammalian protein that binds symmetrically methylated CpG sequences and regulates gene expression in association with DNA methylation. This protein possesses a conserved sequence, named methyl-CpG binding domain (MBD), among a family of methyl-CpG binding proteins that mediate the biological consequences of the methylation. In addition, MBD1 has at least five isoforms due to alternative splicing events, resulting in the presence of CXXC1, CXXC2, and CXXC3 in MBD1 isoforms v1 (MBD1v1) and MBD1v2, and CXXC1 and CXXC2 in MBD1v3 and -v4. In the present study, we have investigated the significance of MBD, CXXC, and the C-terminal transcriptional repression domain (TRD) in MBD1. A bacterially expressed MBD binds efficiently to densely methylated rather than to sparsely methylated DNAs. In both methylation-deficient *Drosophila melanogaster* SL2 cells and mammalian CHO-K1 cells, MBD1v1 represses transcription preferentially from both unmethylated and sparsely methylated promoters, while MBD1v3 inhibits densely methylated but not unmethylated promoter activities. The CXXC3 sequence in MBD1v1 is responsible for the ability to bind unmethylated promoter. Furthermore, we have constructed mutant-type MBD1s in which the functionally important residues Arg22, Arg30, Asp32, Tyr34, Arg44, Ser45, and Tyr52 are changed to alanine to investigate the correlation between the structure and function of the MBD in MBD1. Excepting those for Ser45 and Tyr52, none of the recombinant MBD mutants bound to the densely methylated or unmethylated DNAs, and green fluorescent protein-fused MBD1 mutants did not localize properly in the nucleus. All the MBD1v1 and -v3 mutants lost the activity of methylation-dependent gene repression. Based on these findings we have concluded that MBD1 acts as a transcriptional regulator depending on the density of methyl-CpG pairs through the cooperation of MBD, CXXC, and TRD sequences.

DNA methylation at CpG dinucleotides is the major epigenetic modification of mammalian genomes and is required for gene regulation and genome stability (1, 2, 5, 19, 30, 39, 40). *trans*-acting factors such as the methyl-CpG binding proteins, termed MeCPs, are involved in methylation-based gene repression and affect chromatin structure (9, 16, 26, 31). MeCP1 complex and MeCP2 were initially reported to bind specifically to methylated DNAs (18, 22) and repress transcription by recruiting histone deacetylases and corepressor proteins (15, 25, 28). An additional four MeCP members, methyl-CpG binding domain 1 (MBD1), MBD2, MBD3, and MBD4 (also known as MED1), have been identified based on conserved amino acid sequences homologous to the MBD of MeCP2 (8, 13). These MBD-containing proteins can bind selectively to methyl-CpG pairs, although MBD3 may have a differential affinity for methylated DNA (37, 41). MBD2 is a transcriptional repressor which is associated with histone deacetylase in the MeCP1 complex in mammalian cells (28). MBD3 also regulates transcription by forming a Mi-2–NuRD complex with nucleosome remodeling and histone deacetylase activities in *Xenopus laevis* and mammalian cells (37, 41). The Mi-2–NuRD complex does not bind directly to methylated DNA but is tethered to it and stabilized by the presence of MBD2. MBD4, which is mutated in human carcinomas with microsatellite instability, is a thymine glycosylase that recognizes the product of deamination at methyl-CpG sites, as a part of the DNA repair system (3, 14, 32). Among the MeCP family of proteins, MBD1 is characterized by sequences similar to a cysteine-rich CXXC domain which was originally found in DNA methyltransferase (4) and *trithorax* group protein ALL-1 (also known as HRX) (21). Recently, we have reported the presence of at least five MBD1 isoforms, including MBD1v1, MBD1v2, MBD1v3, and MBD1v4, which are alternatively spliced in the region of the CXXC domains and the C terminus (10). MBD1v1 and MBD1v2, which contain three CXXC motifs (CXXC1, CXXC2, and CXXC3), may repress gene expression from both unmethylated and methylated promoters. In contrast, MBD1v3 and MBD1v4, which contain two CXXC motifs (CXXC1 and CXXC2), appear to inhibit transcription only when the promoters are methylated. Thus, MBD1 isoforms play multiple roles in gene regulation, although the significance of the two or three CXXC domains remains to be elucidated. By nuclear magnetic resonance (NMR) spectroscopic analysis, we have demonstrated that the MBD of MBD1 folds into a novel α-β sandwich structure with characteristic loops (29). Three basic residues, Arg22, Arg30, and Arg44, may form a positively charged surface for the DNA contact, and the MBD is suggested to interact with a methyl-CpG pair at residues Tyr34 and Asp32, which are conserved among the MeCP members. The structure of the MBD of MBD1 is analogous to that of MeCP2 which has been reported by Wakefield et al. (38), suggesting the validity of this MBD-methylated DNA binding model. In the present study, we have investigated the

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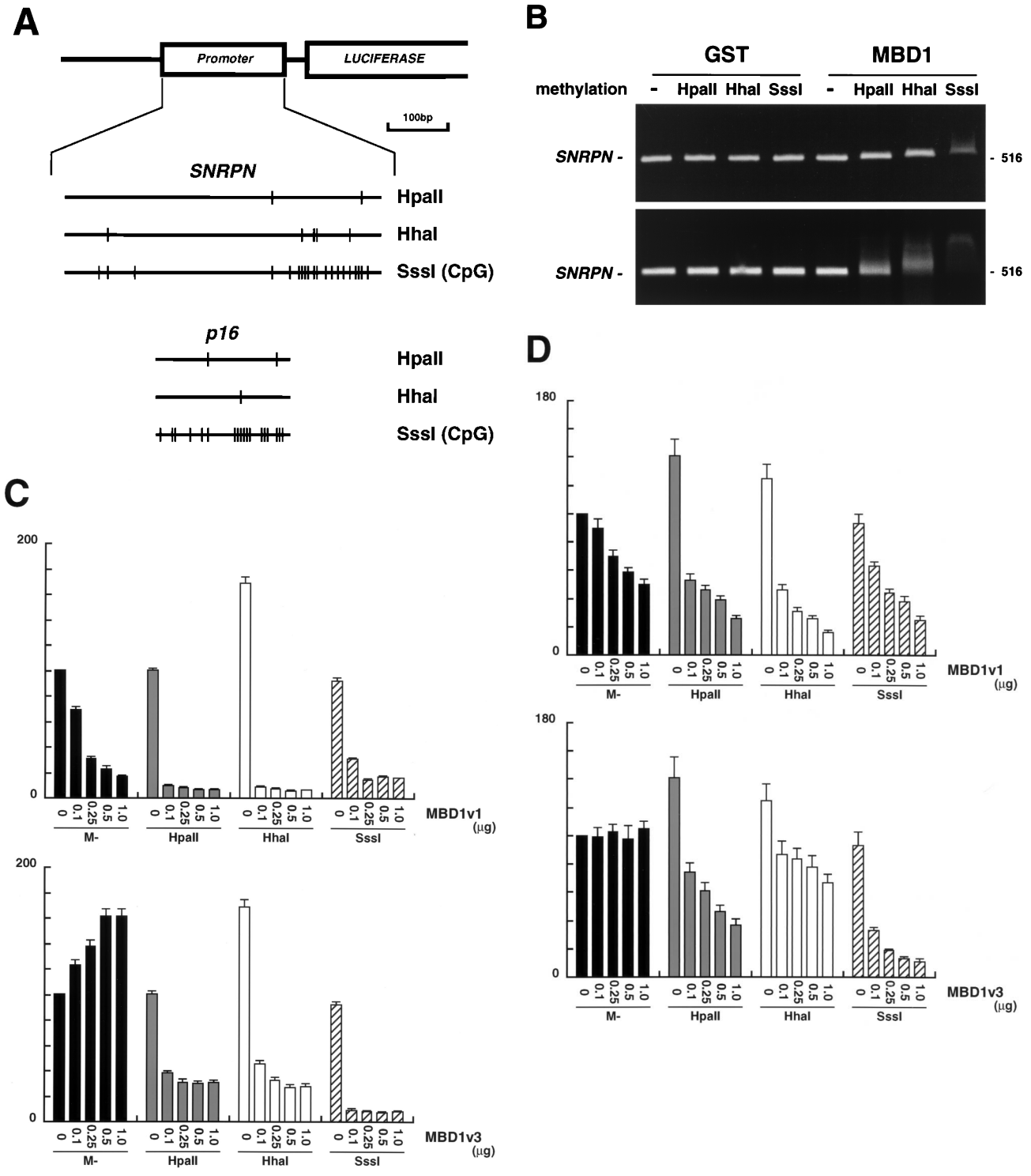


FIG. 1. Effect of MBD1 isoforms on methylated and unmethylated promoters. (A) PCR-amplified DNA fragments from human imprinted *SNRPN* and tumor suppressor *p16* genes were used for a band shift analysis and subcloned upstream of a luciferase cDNA in a pGL3-Basic vector. The PCR fragments and pGL3 constructs were methylated in vitro using *Hpa*II, *Hha*I, and *Sss*I (CpG) methyltransferases. The methyl-CpG sites modified by these enzymes are shown by vertical lines. (B) Band shift of methylated DNA complexed with the methyl-CpG binding domain of MBD1. Unmethylated (−) and methylated fragments containing *SNRPN* promoter were incubated with MBD1 (residues 1 to 75) or GST. In the upper and lower panels, the amount of the protein incubated with DNA fragments was 0.5 and 1.0 μg, respectively. (C and D) Regulation of Sp1-activated transcription by MBD1v1 and -v3 in *Drosophila* SL2 cells (*SNRPN* [C] or *p16* [D]). Unmethylated (M-) or *Hpa*II-, *Hha*I-, or *Sss*I-methylated promoter-inserted pGL3 vector (0.5 μg) was cotransfected with Sp1-expressing plasmid pPacSp1 (0.5 μg), MBD1-expressing plasmids (pAc5.1-MBD1v1 and pAc5.1-MBD1v3) (0 to 1.0 μg), and insertless plasmid pAc5.1/V5-His (mock) (1.0 to 0 μg). The luciferase activity of unmethylated pGL3 in combination with pPacSp1 and 1.0 μg of pAc5.1/V5-His (mock) was normalized to 100, and the relative luciferase activities (means + standard deviations [error bars]) were determined after correcting the transfection efficiency by pAc5.1-pRL (0.1 μg). (E) Detection of endogenous MBD1 by an antibody raised against the recombinant MBD1. MBD1 was found to be approximately 80 kDa in HeLa and A549 cells but not in SL2 and CHO-K1 cells.

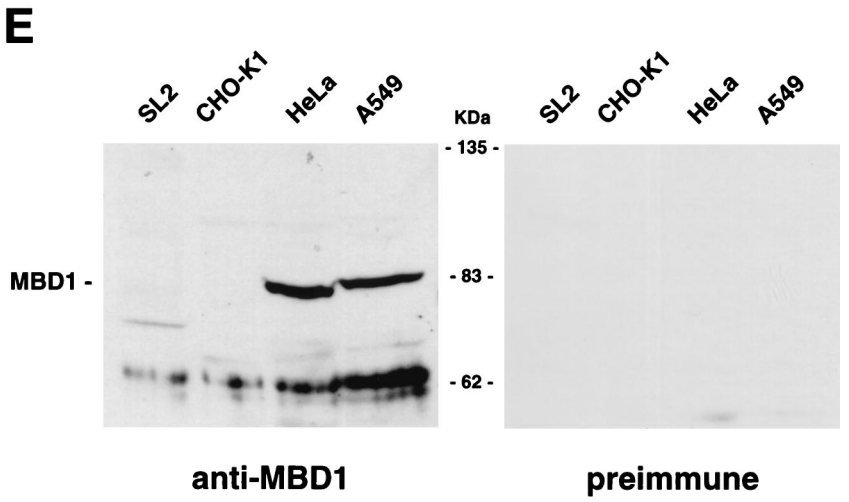


FIG. 1—*Continued*.

functional roles of both MBD, CXXC, and the C-terminal transcriptional repression domain in MBD1 relative to the status of DNA methylation. Further, the correlation between the NMR structure and function of the MBD is demonstrated to facilitate our understanding of the molecular interaction between the MeCPs and the genome methylation.

MATERIALS AND METHODS

Cell lines and cultures. HeLa and A549 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 nutrient medium (Gibco BRL, Rockville, Md.) supplemented with 5% or 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, Md.). CHO-K1 cells were grown in Ham's F-12 nutrient medium (Gibco BRL) supplemented with 10% (vol/vol) heat-inactivated FBS. Schneider cell line 2 (SL2) derived from *Drosophila melanogaster* embryos was cultured in Schneider's *Drosophila* medium (Gibco BRL) with 10% (vol/vol) heat-inactivated FBS (Gibco BRL) and 2 mM glutamine.

Confocal laser scanning microscopic analysis. The HeLa cells were incubated for 24 h at 37°C after the transfection of green fluorescent protein (GFP)-fused MBD1 expression vectors. After being washed two times with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde in PBS for 10 min. After a wash with PBS, the samples were mounted in 80% glycerol. The cells were visualized using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Western blot analysis. Samples containing equal amounts of protein (15 μg) from the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at 4°C with PBS containing 10% nonfat dry milk and then incubated with rabbit anti-MBD1 polyclonal antibody (10), mouse anti-GAL4 monoclonal antibody, or rabbit anti-GFP polyclonal antibody (Santa Cruz, Santa Cruz, Calif.) in PBS containing 0.03% Tween 20 for 1 h. After being washed with PBS containing 0.3% Tween 20, the membrane was incubated with species-appropriate horseradish peroxidase-conjugated secondary antibody for 40 min. After the membrane had been washed again with PBS containing 0.3% Tween 20, visualization was performed using an enhanced chemiluminescence detection system (Amersham, Little Chalfont Buckinghamshire, England).

Construction of GST-fused MBD1 protein and band shift assay. Full-length MBD1v1 and MBD1v3, MBD1v1 (amino acids 62 to 605), and MBD1v3 (amino acids 62 to 549) were expressed using a pGEX-2TH bacterial expression vector. These glutathione *S*-transferase (GST)-fused MBD1 proteins were purified as described previously (10). The *MBD1v1* cDNA fragments (amino acids 1 to 84) were also cloned into pGEX-2TH, and seven mutant constructs (R22A, R30A, R32D, Y34A, R44A, S45A, and Y52A) were prepared with a site-directed mutagenesis. These GST-fused MBD1 proteins were expressed and purified, and MBD1 (amino acids 1 to 75) was obtained and referred to as MBD1 MBD (29). DNA fragments (516 bp long) for the promoter region of the human *SNRPN* gene were amplified from human genomic DNA (10) and were methylated with *Hpa*II, *Hha*I, or *Sss*I methyltransferases as indicated by the manufacturer (New England Biolabs, Beverly, Mass.). For a band shift assay, the unmethylated or methylated DNA fragments (0.2 μg each) were incubated with one of the MBD1 proteins or GST (0.5 or 1.0 μg each) in a binding buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.4% glycerol, and 0.1% Triton X-100 on ice for 30 min. The DNA-protein complexes were then electrophoresed on 1.5% agarose gels and were stained with ethidium bromide.

Construction of expression plasmids. The full-length cDNAs for *MBD1v1* and *MBD1v3* were ligated into a pCGN mammalian expression vector (termed pCGN-MBD1v1 and pCGN-MBD1v3), into a pEGFP-C1 expression vector (termed pEGFP-MBD1v1 and pEGFP-MBD1v3) (Clontech, Palo Alto, Calif.), and into a pAc5.1/V5-His *D. melanogaster* expression vector (termed pAc5.1-MBD1v1 and pAc5.1-MBD1v3) (Invitrogen, Carlsbad, Calif.) (10). MBD1v1 and MBD1v3 with deletions of the MBD (amino acids 1 to 61) were cloned into the *Eco*RV and *Xba*I sites of the pAc5.1/V5-His vector (termed pAc5.1-MBD1v1ΔN and pAc5.1-MBD1v3ΔN), into the *Xba*I site of the pCGN vector (termed pCGN-MBD1v1ΔN and pCGN-MBD1v3ΔN), and into the *Xba*I site of the pEGFP-C1 vector (termed pEGFP-MBD1v1ΔN and pEGFP-MBD1v3ΔN). Four deletion mutants of MBD1v1, named MBD1v1Δ1, MBD1v1Δ2, MBD1v1Δ3, and MBD1v1Δ4, were constructed in the pAc5.1/V5-His vector: the deleted regions are amino acids 113 to 222, 219 to 320, 328 to 421, and 113 to 605, respectively. The *MBD1* cDNAs (amino acids 1 to 89) for the wild type and seven mutants (R22A, R30A, R32D, Y34A, R44A, S45A, and Y52A) were ligated into a pEGFP-C1 vector [termed pEGFP-wt. MBD1 and pEGFP-MBD1(R22A) to pEGFP-MBD1(Y52A) (mutant shown in parentheses)] and the *MBD1* cDNAs (amino acids 1 to 61) were cloned into the *Eco*RV site of pAc5.1-MBD1v1ΔN and pAc5.1-MBD1v3ΔN [termed pAc5.1-wt. MBD1v1, pAc5.1-wt. MBD1v3, pAc5.1-MBD1v1(R22A) to pAc5.1-MBD1v1(Y52A), and pAc5.1-MBD1v3(R22A) to pAc5.1-MBD1v3(Y52A)]. To express epitope-tagged MBD1, the pCGN-MBD1 and pEGFP-MBD1 vectors were transfected into CHO-K1 or HeLa cells by the liposome-mediated gene transfer method. The pAc5.1-MBD1 vectors were transfected to *Drosophila* SL2 cells by the calcium phosphate method.

The GAL4 cDNA (amino acids 1 to 147), which was amplified from pBIND vector (Promega), was ligated into the *Hind*III and *Eco*RV sites of pcDNA3 vector (termed pCMV-GAL4). The resulting plasmid was used to make constructs capable of expressing fusions between the GAL4 DNA binding domain and portions of MBD1. Amplified fragments from MBD1 were cloned into the *Eco*RV and *Not*I sites of pCMV-GAL4. The *Kpn*I-*Nhe*I fragment containing five GAL4 binding sites from pG5*luc* vector (Promega) was inserted into the *Kpn*I-*Nhe*I site of the *SNRPN* promoter-inserted pGL3-Basic vector.

Luciferase assay. The *SNRPN*- and *p16* promoter-inserted pGL3-Basic vector was either unmethylated or methylated by one of the methyltransferases (10). At 48 h after the cotransfection of the promoter-inserted pGL3, pCGN-MBD1, and pRL-SV40 (Promega), which was used for monitoring the transfection efficiency, the CHO-K1 or HeLa cells were collected and lysed by a lysis buffer offered by the manufacturer (Promega). For *Drosophila* cells, we cotransfected the promoter-inserted pGL3, pAc5.1-MBD1, an Sp1 expression plasmid pPacSp1 (7), and pAc5.1-pRL for checking the transfection efficiency. The insertless pCGN, pAc5.1/V5-His, and pCMV-GAL4 were used as mock vectors. The luciferase activities were determined with a dual-luciferase reporter assay system and a luminometer (Promega). Values are given as the means and standard deviations of results from three independent experiments.

RESULTS

Association of MBD1 isoforms with methylated and unmethylated promoters. To investigate the function of MBD1,

we utilized promoter-associated CpG islands from human genes, imprinted *SNRPN* (34), and tumor suppressor *p16* as described previously (10) (Fig. 1A). The nucleotide component of the *SNRPN* DNA was 516 bp long (G + C content, 52.0%; CpG/GpC = 0.43). Two, five, and twenty sites within the promoter sequence were modified by *Hpa*II, *Hha*I, and *Sss*I (CpG) methyltransferases, and methylation densities with the use of these enzymes are 0.4, 1.0, and 3.9 methyl-CpGs/100 bp, respectively. On the other hand, the component of the *p16* DNA was 221 bp long (G + C content, 70.1%; CpG/GpC = 0.69). Two, one, and eighteen sites within the sequence were methylated by *Hpa*II, *Hha*I, and *Sss*I (CpG) methyltransferases, and methylation densities are 0.9, 0.45, and 8 methyl-CpGs/100 bp, respectively. The MBD1 (amino acids 1 to 75 containing the MBD) and GST were incubated with the PCR-amplified fragment from the *SNRPN* promoter which had been either unmethylated or methylated by each of the methyltransferases in vitro. A band shift analysis was performed by agarose gel electrophoresis (Fig. 1B). MBD1 bound easily to *Sss*I-methylated DNA, and the DNA-MBD1 complex was found to be shifted to a slow-migrating, higher-molecular-mass band. MBD1 bound to DNAs methylated sparsely with *Hpa*II and *Hha*I methyltransferases, but it did not associate with the unmethylated version. GST bound to neither unmethylated nor methylated DNAs. To observe the selective association of MBD1 with methylated DNAs, the amount of the protein in each lane, 0.5 and 1.0 μg, was used in the upper and lower panels, respectively. This finding indicates that the MBD of MBD1 binds efficiently to a methyl-CpG in a dose-dependent manner. Next, to elucidate whether full-length MBD1 isoforms regulate gene activity in a methylation-dependent manner, a luciferase reporter assay was performed in *D. melanogaster* SL2 cells, which lack genome methylation (20, 36), as a host cell. *Drosophila* cells possess a general transcription machinery homologous to that of mammalian cells (11, 12), but the methylation-insensitive transcription factor Sp1 (7) and endogenous MeCPs are known to be deficient (10, 17, 29, 35, 37). We utilized *SNRPN* promoter-inserted pGL3 vector (pGL3-SNRPN) and *p16* promoter-inserted pGL3 vector (pGL3-p16) (Fig. 1A), which have one and three Sp1 binding motifs, respectively, to express a *Photinus pyralis* luciferase under the control of the promoter. The promoter-inserted pGL3 vector was methylated by either *Hpa*II, *Hha*I, or *Sss*I methyltransferases, and the methylation status has been already proved to be stably maintained in the *Drosophila* cells (10, 17). pGL3-SNRPN or pGL3-p16, Sp1 expression vector pPacSp1, and pAc5.1-MBD1 or insertless pAc5.1/V5-His were cotransfected into *Drosophila* cells, and the level of luciferase activity was measured with a luminometer (Fig. 1C and D). The pAc5.1-pRL vector expressing *Renilla reniformis* luciferase was simultaneously used as an internal control for correcting the transfection efficiency. In this report, we chose the expression of MBD1v1 and MBD1v3, whose protein structures are identical except for the presence of CXXC3 in MBD1v1 (see Fig. 4A). Cotransfection of pPacSp1, together with pGL3-SNRPN or pGL3-p16, led to approximately 10- to 40-fold increases in the promoter activity from unmethylated and *Hpa*II-, *Hha*I-, and *Sss*I-methylated constructs, in comparison with cotransfection of insertless plasmid A5C instead of pPacSp1 (data not shown). The relative luciferase activity of the methylated constructs had an increase similar to that of the unmethylated version, indicating that Sp1 can transactivate both *SNRPN* and *p16* promoters in *Drosophila* cells, even when the promoters are methylated (Fig. 1C and D). The expression of MBD1v1 and -v3 repressed all of the methylated constructs in a dose (MBD1)-dependent manner. In addition, MBD1v1 also inhibited transcription from the unmethylated promoter, whereas the unmethylated promoter activity was not inhibited by MBD1v3. Interestingly, MBD1v1 and -v3 tended to repress transcription preferentially from sparsely (*Hpa*II and *Hha*I) and densely (*Sss*I) methylated promoters by about 10-fold, respectively. Thus, MBD1 isoforms can control the Sp1-activated transcription from unmethylated, hypomethylated, and hypermethylated promoters.

Furthermore, we investigated the abundance of endogenous MBD1, using an antibody against a GST-fused human MBD1 (amino acids 1 to 421, containing the MBD and CXXC domains). The antibody is expected to cross-react with the MBD1 homolog in different species. A Western blot analysis indicated that an approximately 80-kDa band for MBD1 is present in HeLa and A549 cells but not in SL2 and CHO-K1 cells (Fig. 1E). In contrast, a preimmune antibody did not detect any bands in the same cell lysates. Therefore, SL2 and CHO-K1 cells were chosen for the transfection assays of MBD1 in this study.

Transcriptional regulation by MBD1 isoforms in mammalian cells. In order to analyze whether MBD1 isoforms affect gene expression according to the status of DNA methylation, we further employed mammalian CHO-K1 cells. pCGN-MBD1v1 and pCGN-MBD1v3 for expressing full-length MBD1v1 and -v3, and pCGN-MBD1v1ΔN and pCGN-MBD1v3ΔN for expressing MBD1v1 and -v3 with deletions of MBD (amino acids 1 to 61), respectively, were transfected into CHO-K1 cells, and a Western blot analysis was performed to confirm their expression (data not shown). The unmethylated or methylated pGL3-SNRPN or pGL3-p16, pCGN-MBD1 or insertless mock pCGN, and pRL-SV40 vectors were cotransfected into the cells, and the level of luciferase activity was measured (Fig. 2). In the mock transfections, the relative luciferase activity of the *Hpa*II-, *Hha*I-, and *Sss*I-methylated pGL3-SNRPN was repressed by approximately 3-, 18-, and 200-fold compared with the unmethylated version respectively (Fig. 2A and B). The relative luciferase activity of the *Hpa*II- and *Sss*I-methylated pGL3-p16 was repressed by approximately 5- and 300-fold compared with the unmethylated version, respectively (Fig. 2C and D). These are due to the involvement of endogenous MeCPs and other cellular factors (5, 26, 33). However, the *Hha*I-methylated pGL3-p16, which possesses a single methyl-CpG site in the promoter, did not decrease the luciferase activity. The expression of MBD1v1 strongly repressed transcription from both the unmethylated and methylated constructs even at low levels of pCGN-MBD1v1 (0.1 μg), and MBD1v1 produced the most-efficient silencing effect on sparsely methylated promoters (Fig. 2A and C). MBD1v1ΔN lacking the MBD also moderately suppressed unmethylated and *Hpa*II- and *Hha*I-methylated versions, while transcription from the *Sss*I-methylated promoter was somewhat enhanced, by three- to fivefold, as observed later in *Drosophila* cells (see Fig. 5C). On the other hand, neither MBD1v3 nor MBD1v3ΔN inhibited the activities of unmethylated or *Hpa*II- or *Hha*I-methylated promoters, and MBD1v3 repressed transcription only from the *Sss*I-methylated construct (Fig. 2B and D). The small difference in the effect of MBD1 on the two promoters may depend upon the status of methyl-CpGs relative to the transcription factor motifs in their sequences. Thus, MBD1v1 and -v3 preferentially repress transcription from sparsely and densely methylated promoters, respectively, in both *Drosophila* and mammalian cells.

Effect of each CXXC domain in MBD1 on Sp1-activated transcription. To demonstrate that MBD1v1 and -v3 distinctly regulate transcription through the CXXC domains, four deletion mutants of MBD1v1 (MBD1v1Δ1, MBD1v1Δ2, MBD1v1Δ3, and MBD1v1Δ4) were constructed in the pAc5.1/V5-His vec-

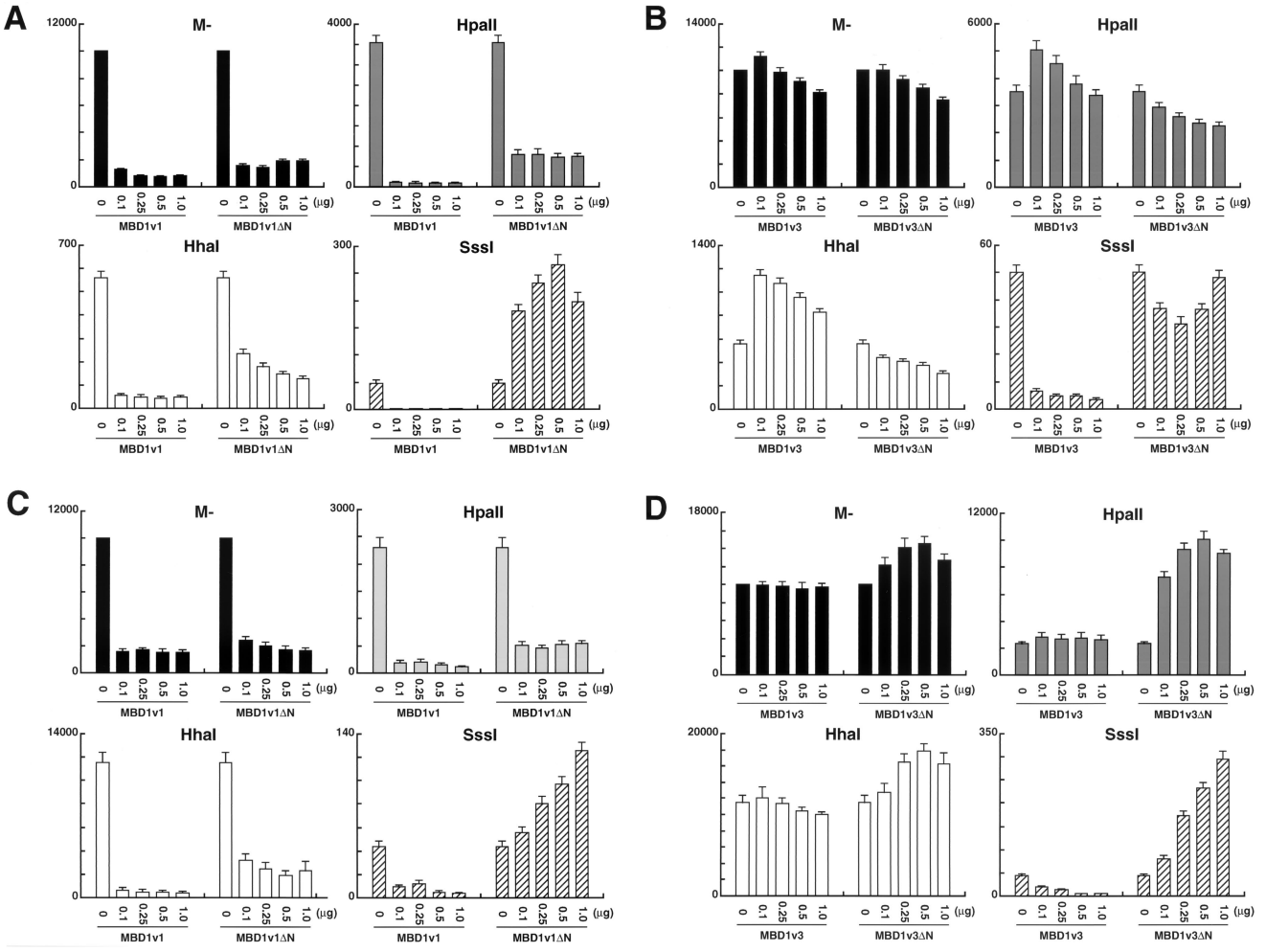


FIG. 2. Transcriptional regulation by MBD1 isoforms and their mutants with deletions of the methyl-CpG binding domain in mammalian CHO-K1 cells. Unmethylated or *Hpa*II-, *Hha*I-, or *Sss*I-methylated promoter-inserted pGL3 vector (0.5 μg) was cotransfected with MBD1-expressing plasmids (pCGN-MBD1v1, pCGN-MBD1v3, pCGN-MBD1v1ΔN, and pCGN-MBD1v3ΔN) (0 to 1.0 μg) and insertless plasmid pCGN (mock) (1.0 to 0 μg). The luciferase activity of unmethylated pGL3 in combination with 1.0 μg of pCGN (mock) was normalized to 10,000, and the relative luciferase activities (means + standard deviations [error bars]) were determined after correcting the transfection efficiency by pRL-SV40 (0.1 μg). The combinations of pGL3-SNRPN and pCGN-MBD1v1 or pCGN-MBD1v1ΔN (A), pGL3-SNRPN and pCGN-MBD1v3 or pCGN-MBD1v3ΔN (B), pGL3-p16 and pCGN-MBD1v1 or pCGN-MBD1v1ΔN (C), and pGL3-p16 and pCGN-MBD1v3 or pCGN-MBD1v3ΔN (D) are shown.

tor: the deleted regions are residues 113 to 222, containing CXXC1; residues 219 to 320, containing CXXC2; residues 328 to 421, containing CXXC3; and residues 113 to 605, containing CXXC1 to CXXC3 and the C terminus, respectively (Fig. 3A). They were each transfected into *Drosophila* SL2 cells together with pPacSp1, pAc5.1-pRL, and either unmethylated or methylated pGL3-SNRPN (Fig. 3B). Full-length MBD1v1, MBD1v1Δ1, and MBD1v1Δ2 inhibited both unmethylated and methylated promoter activities. In contrast, MBD1v1Δ3 repressed transcription from the *Sss*I-methylated promoter and diminished the suppressive effect on *Hpa*II- and *Hha*I-methylated promoters. The result that MBD1v1Δ3 rather activated transcription from the unmethylated promoter is consistent with the data for MBD1v3 shown in Fig. 1C. Taken together, we conclude that the CXXC3 domain in MBD1v1 is responsible for the repression of unmethylated promoter, regardless of the presence of the MBD. In addition, the smallest mutant, MBD1v1Δ4, containing the MBD and nuclear localization signal (NLS) sequences, moderately reduced the methylated promoter activities. This suggests that the repression of methylated promoters by MBD1 depends partly on the promoter occupation through the contact between the MBD and methylated DNA.

Mutagenesis of functionally important residues in the MBD of MBD1. A series of MBD1 mutants were prepared to investigate the interaction between the MBD and methylated promoter (Fig. 4A). The following seven residues were chosen for the mutagenesis, based on the data from the NMR structure, chemical shift perturbation, and sequence homology among the MeCPs: Arg22, Arg30, Asp32, Tyr34, Arg44, Ser45, and Tyr52 (29). All point mutations were to alanine, and these mutants did not disrupt the native tertiary structure of the MBD. In the upper panel of Fig. 4B, these MBD mutants fused to GFP were equally expressed in HeLa cells and used in Fig. 5B. In the lower panels, MBD1v1 and -v3 and their mutants produced from a pAc5.1/V5-His vector were also equally expressed in *Drosophila* SL2 cells for the assay in Fig. 5C. In MBD1v1ΔN and MBD1v3ΔN, the MBD sequence (amino acids 1 to 61) was deleted, and wild-type and point-mutated

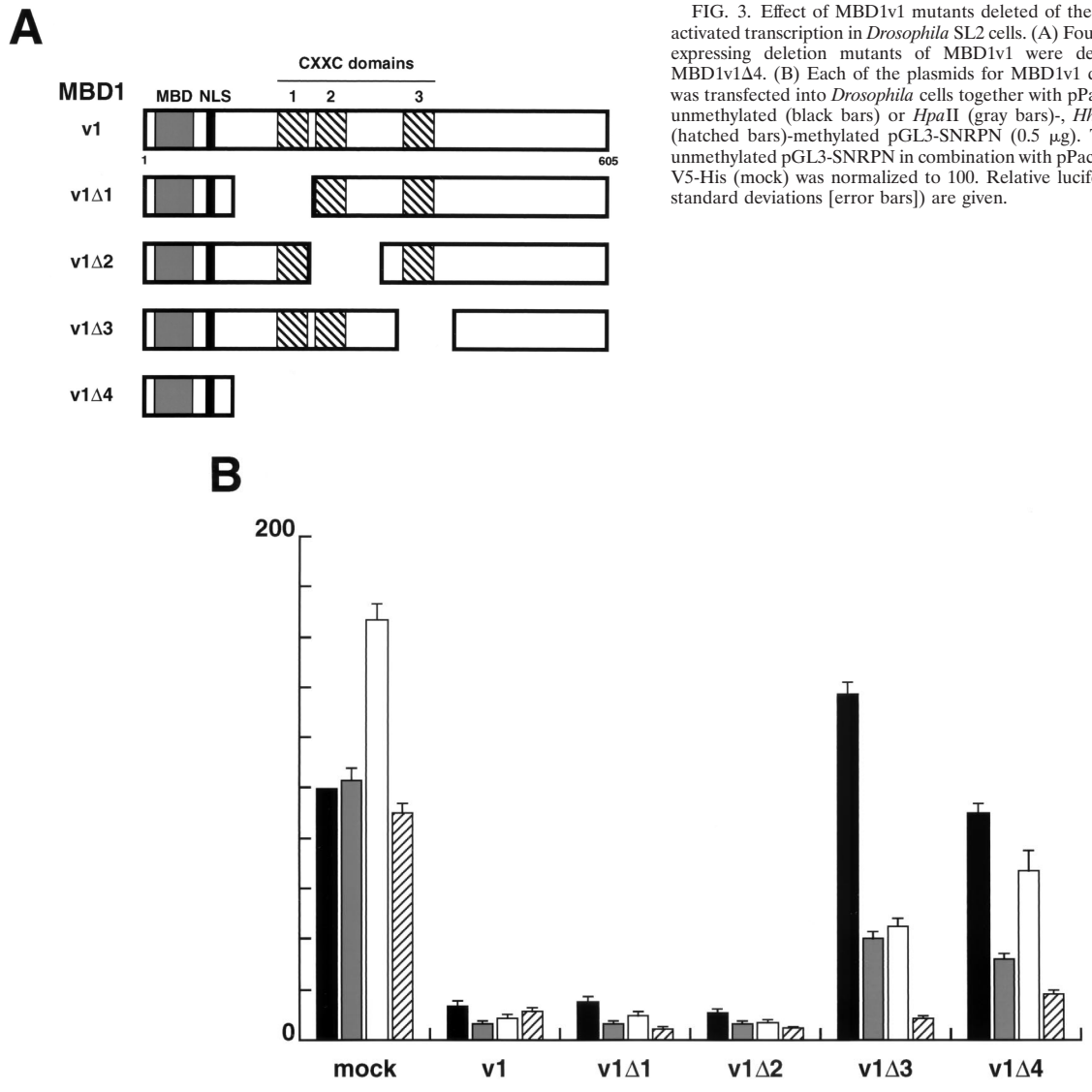


FIG. 3. Effect of MBD1v1 mutants deleted of the CXXC domains on Sp1-activated transcription in *Drosophila* SL2 cells. (A) Four pAc5.1/V5-His plasmids expressing deletion mutants of MBD1v1 were designated MBD1v1Δ1 to MBD1v1Δ4. (B) Each of the plasmids for MBD1v1 deletion mutants (1.0 μg) was transfected into *Drosophila* cells together with pPacSp1 (0.5 μg), and either unmethylated (black bars) or *Hpa*II (gray bars)-, *Hha*I (white bars)-, or *Sss*I (hatched bars)-methylated pGL3-SNRPN (0.5 μg). The luciferase activity of unmethylated pGL3-SNRPN in combination with pPacSp1 and 1.0 μg of pAc5.1/V5-His (mock) was normalized to 100. Relative luciferase activities (means + standard deviations [error bars]) are given.

MBD1v1 and -v3 were constructed concurrently by the same experimental procedure.

Alteration of DNA binding, intranuclear localization, and methylation-mediated transcriptional silencing in MBD1 mutants. The site-directed MBD mutants (amino acids 1 to 75) from MBD1 were constructed in *E. coli*, and their abilities to bind unmethylated and *Sss*I-methylated *SNRPN* promoter were examined by band shift analysis (Fig. 5A). Wild-type MBD1 bound preferentially to methylated DNA, and the DNA-MBD1 complex was found to be shifted to a slow-migrating band. Five (R22A, R30A, D32A, Y34A, and R44A) of the seven mutants markedly lost their ability to bind methylated DNA. This result agrees with our NMR data showing that these residues are mainly involved in the methylated DNA binding (29). Two mutants, S45A and Y52A, bound to the methylated version to some extent. None of the MBD mutants tested here associated with the unmethylated DNA. Our previous report indicated that the use of quantitatively increased amounts of MBD mutants gave a very similar result (29). Next, we visualized the intranuclear localization of GFP-fused MBD1 mutants in intact HeLa cells, using a confocal laser scanning microscope (Fig. 5B). MBD1v1, MBD1v3, and wild-type MBD1 (amino acids 1 to 89 containing the MBD and NLS) showed a punctate distribution in the interphase nuclei except for the nucleolus, and multiple foci of different sizes with intense staining were seen in the nuclei of transfected cells. This localization of MBD1 is demonstrated to depend mostly on genome methylation (10). MBD1v1ΔN, MBD1v3ΔN, and MBD1 mutants (R22A, D32A, R44A) were distributed nonspecifically throughout the nuclei, and they localized uniformly in the nucleolus. Interestingly, mutants S45A and Y52A presented a punctate labeling of the nuclei, although the formation of foci was significantly inhibited. In addition, mutants R30A and Y34A, whose expression levels were similar to those of other mutants, tended to form only a few foci but not to exist in the nucleolus. Thus, these seven residues within the MBD are important for the proper distribution of MBD1 in the nucleus as well as for the binding to methylated DNA.

Next, we analyzed the functional roles of the MBD in methylation-mediated transcriptional repression, using a luciferase reporter assay in *Drosophila* cells. *Hha*I- or *Sss*I-methylated pGL3-SNRPN, Sp1-expressing pPacSp1, and one of the

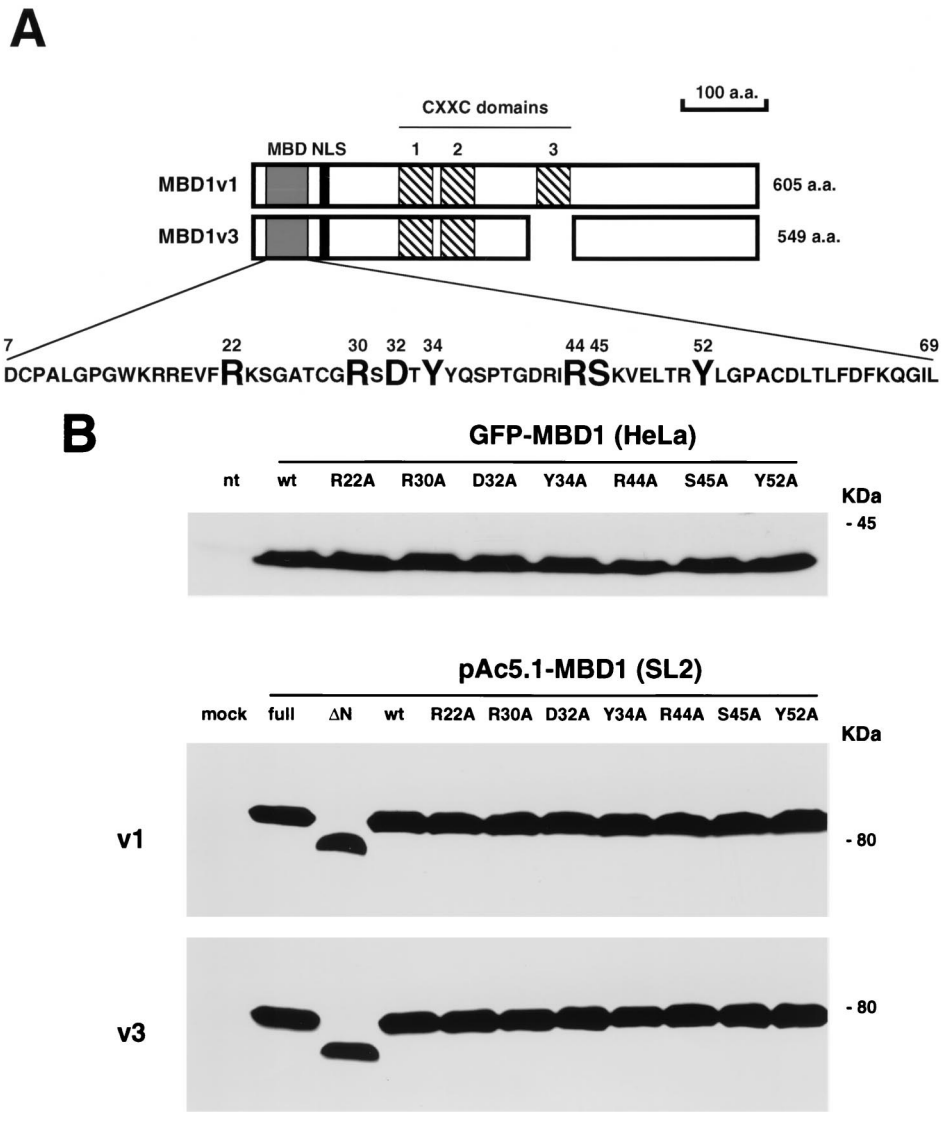
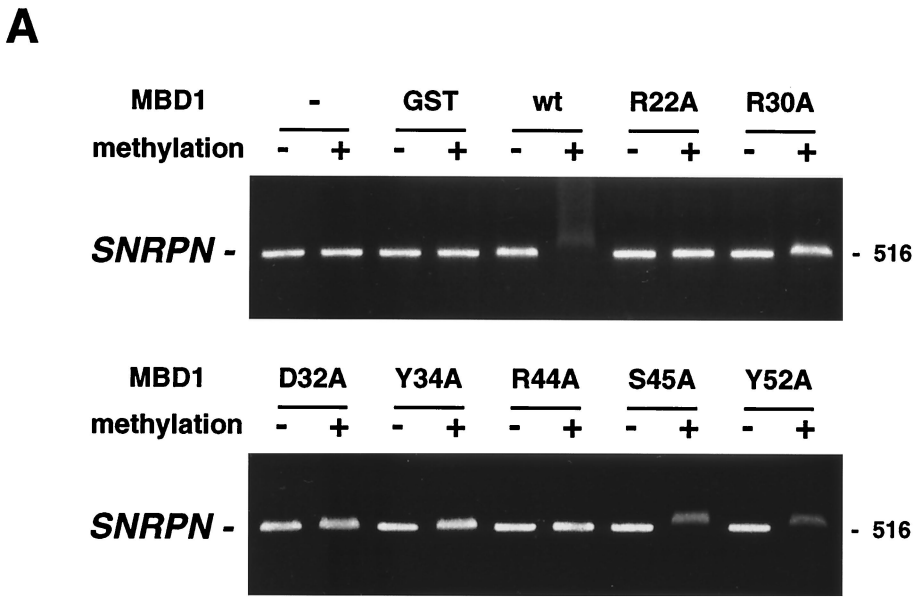


FIG. 4. Site-directed mutagenesis of the methyl-CpG binding domain in MBD1. (A) Diagram of MBD1v1 and -v3. MBD1 has an MBD, an NLS, and two or three cysteine-rich CXXC domains due to alternative splicing events. Seven amino acid residues indicated by oversize capital letters are important for the methylated DNA binding, and they were mutated to alanine. The numbers above the residues indicate the positions relative to the N terminus. a.a., amino acids. (B) Expression of wild-type (wt) and mutant MBD1v1 and MBD1v3 in HeLa and *Drosophila* SL2 cells. pEGFP-wt. MBD1 and pEGFP-MBD1(R22A) to pEGFP-MBD1(Y52A) express wild-type and mutant MBD1 fused to GFP in HeLa cells (upper panel). In SL2 cells, pAc5.1-MBD1v1 and pAc5.1-MBD1v3 express full-length MBD1v1 and MBD1v3, while pAc5.1-MBD1v1ΔN and pAc5.1-MBD1v3ΔN express MBD1v1 and MBD1v3 with the MBD (residues 1 to 61) deleted, respectively. pAc5.1-wt. MBD1v1, pAc5.1-wt. MBD1v3, pAc5.1-MBD1v1(R22A) to pAc5.1-MBD1v1(Y52A), and pAc5.1-MBD1v3(R22A) to pAc5.1-MBD1v3(Y52A) express wild-type and mutant MBD1 (lower panel). A Western blot analysis was performed using anti-GFP and anti-MBD1 polyclonal antibodies. The lysates from nontransfected cells (nt) and mock-transfected cells (mock) are used as a control.

MBD1-expressing plasmids (indicated in Fig. 4B) were co-transfected to *Drosophila* cells, and the level of luciferase activity was measured and corrected based on the transfection efficiency (Fig. 5C). The luciferase activity of unmethylated pGL3-SNRPN in the combination of pPacSp1 and pAc5.1/V5-His mock vector was normalized to 100 (data not shown). The expression of full-length MBD1v1 and wild-type MBD1v1 repressed both *Hha*I- and *Sss*I-methylated *SNRPN* promoters, while MBD1v1ΔN and all the MBD1v1 mutants abolished the methylation-dependent gene silencing. In addition, transcriptional activation was achieved by expressing MBD1v1(S45A) in an *Hha*I-methylated promoter, and MBD1v1ΔN and all the MBD1v1 mutants increased transcription from *Sss*I-methylated promoter by approximately 10-fold. Further, full-length MBD1v3 and wild-type MBD1v3 inhibited both the methylated promoters, and MBD1v3ΔN and all of the mutants for MBD1v3 lost their activities for repressing transcription from the methylated promoter as well. The dominant effect of these MBD1 mutants suggests that MBD1 forms certain complexes with either transcription- or chromatin-related factors in the nucleus. Thus, our findings demonstrate the perfect correlation between the structure and function of the MBD in MBD1.

C-terminal TRD and DNA binding activity of CXXC3 in MBD1. To reach a final conclusion concerning the mechanism of transcriptional regulation by MBD1, we investigated whether MBD1 has an active repression domain and whether the CXXC3 of MBD1v1 can interact directly with DNA in vitro. Until now, MeCP2 was known to have the transcriptional


A
MBD1
-
GST
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R22A
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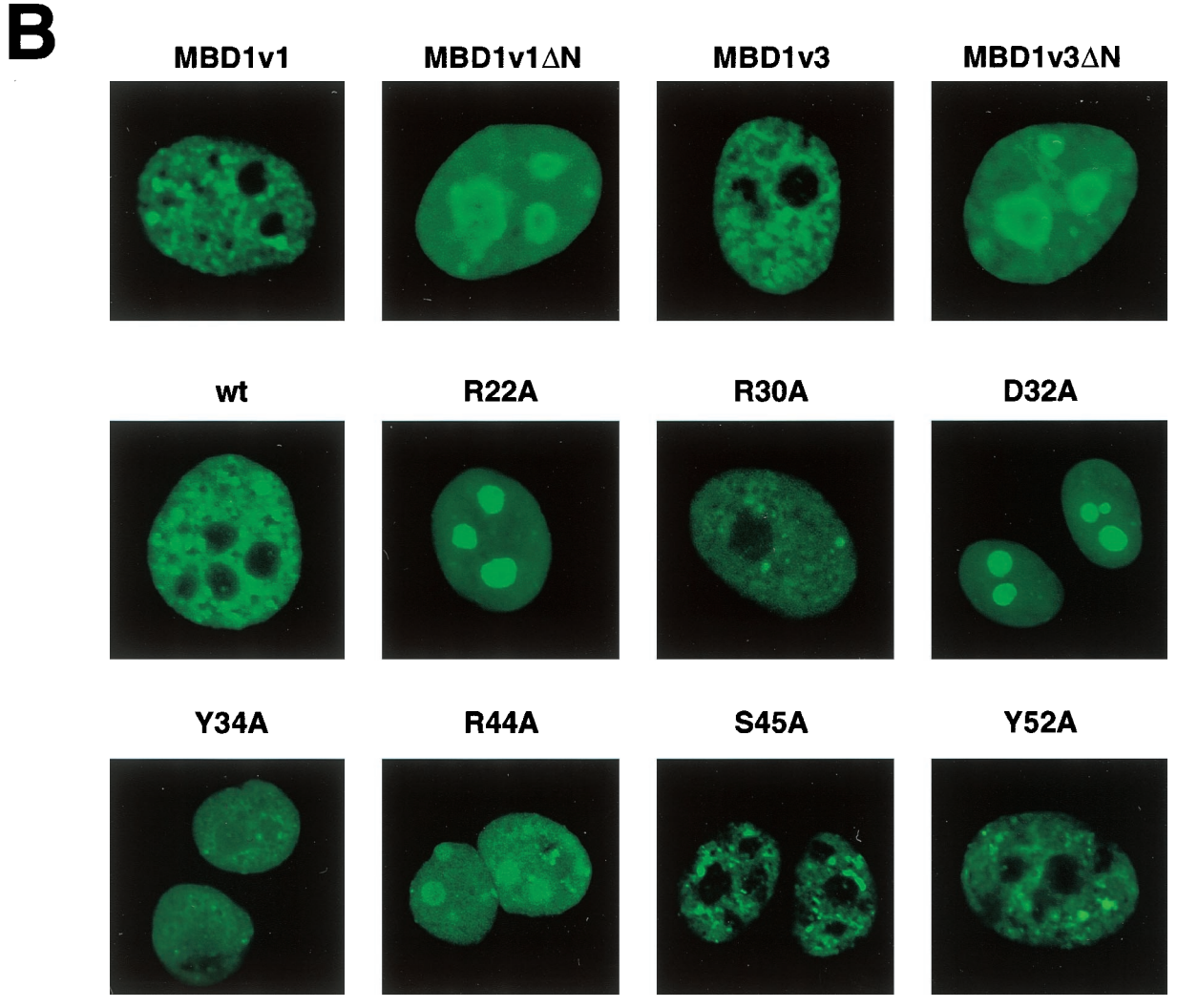
B
MBD1v1
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Y52A

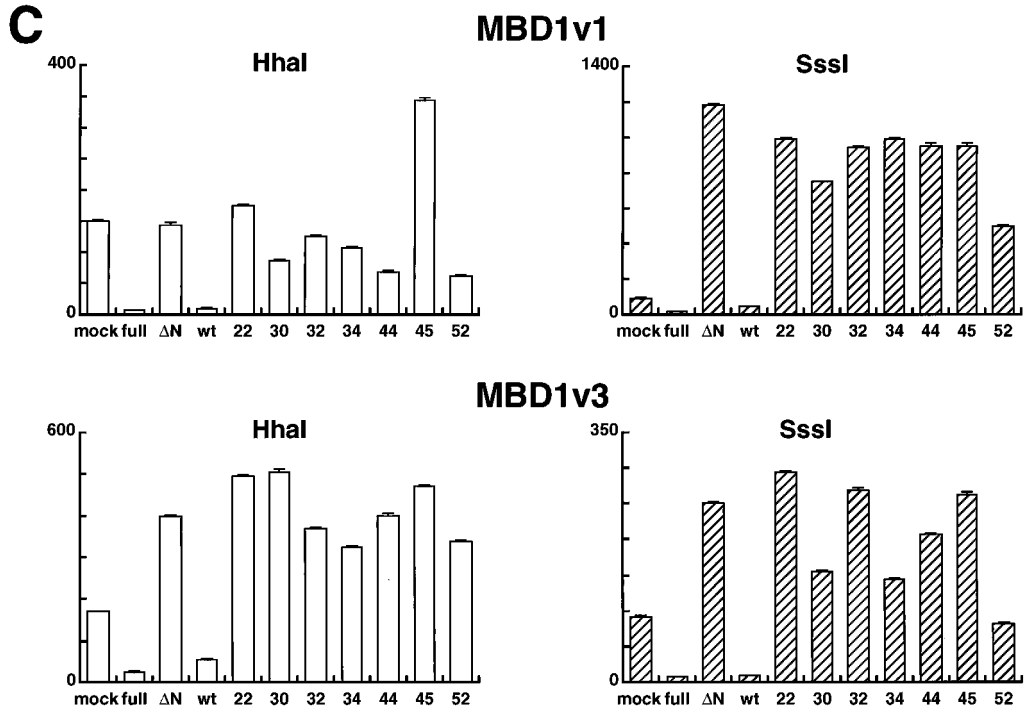

FIG. 5—*Continued*.

repression domain (TRD) and the DNA binding affinity in the C terminus of the protein (6, 23). First, we expressed portions of MBD1 fused to a DNA binding domain of the GAL4 and analyzed the effect of the fusion proteins on a reporter that contains GAL4 binding elements upstream of the *SNRPN* promoter (Fig. 6A). Expression of the effector proteins was found by a Western blot analysis with anti-GAL4 and anti-MBD1 antibodies (data not shown). Eleven GAL4-fusion proteins (termed GAL4-MBD1Δ1 to GAL4-MBD1Δ11) were constructed (Fig. 6B). The TRD which is commonly shared among all MBD1 isoforms was identified in the C-terminal region. MBD1Δ11 is corresponding to the sequence of amino acids 529-592 in MBD1v1. Neither MBD (Δ7) nor any of the CXXC domains (Δ4, Δ5) repressed transcription from the reporter constructs (Fig. 6C).

Next, a band shift analysis was performed to clarify whether the CXXC3 can bind DNA. Full-length MBD1v1 and MBD1v3 and MBD1v1ΔN (amino acids 62 to 605) and MBD1v3ΔN (amino acids 62 to 549) lacking an N-terminal MBD were bacterially expressed as a GST-fused protein (Fig. 6D). Judging from the formation of DNA-MBD1 complex, both full-length MBD1v1 and MBD1v1ΔN were able to bind unmethylated as well as *Sss*I-methylated DNAs in a dose-dependent manner (Fig. 6E). Full-length MBD1v1 showed the most efficient affinity to methylated DNA, suggesting the cooperation of both the MBD and CXXC3 in the complex formation. In contrast, full-length MBD1v3 selectively bound to methylated but not to unmethylated DNA. MBD1v3ΔN associated with neither unmethylated nor methylated versions. These findings demonstrate that the CXXC3 of MBD1v1 can bind directly to both unmethylated and methylated DNAs.

DISCUSSION

In this report, we have presented evidence that MBD1 acts as a transcriptional regulator depending on the density of methyl-CpG pairs through the cooperation of the MBD, CXXCs, and TRD. MBD1v1 and -v3 preferentially repress transcription from sparsely and densely methylated promoters, respectively. Moreover, MBD1v1 can inhibit unmethylated promoter activity via the presence of the CXXC3, while MBD1v3 does not repress transcription from unmethylated promoter.

There are at present five mammalian MeCP family proteins: MBD1, MBD2, MBD3, MBD4, and MeCP2 (13). Each is characterized by the presence of a highly conserved MBD sequence and can bind to symmetrically methylated CpG pairs. The proteins appear to bind the DNA as a monomer, independent of the sequence context outside of the CpG sequence (29, 38). It is noteworthy that the MBD of MBD4 also binds to

FIG. 5. Residues within the methyl-CpG binding domain of MBD1 required for the methylated DNA binding, intranuclear localization, and transcriptional repression of methylated promoter. (A) Band shift of methylated DNA complexed with wild-type (wt) and mutant MBD1. Unmethylated (−) and *Sss*I-methylated (+) DNA fragments of the *SNRPN* promoter were incubated with MBD1 (residues 1 to 75) or GST. (B) Intranuclear localization of GFP-fused MBD1 (full-length and N-terminal deletion ΔN) and MBD1 (residues 1 to 89) with the above-mentioned point mutations. (C) *Hha*I- or *Sss*I-methylated pGL3-SNRPN (0.5 μg) was cotransfected with pPacSp1 (0.5 μg) and one of the MBD1-expressing plasmids [pAc5.1-MBD1v1, pAc5.1-MBD1v1ΔN, pAc5.1-wt. MBD1v1, pAc5.1-MBD1v1(R22A) to pAc5.1-MBD1v1(Y52A), pAc5.1-MBD1v3, pAc5.1-MBD1v3ΔN, pAc5.1-wt. MBD1v3, and pAc5.1-MBD1v3(R22A) to pAc5.1-MBD1v3(Y52A)] (1.0 μg) or insertless plasmid pAc5.1/V5-His (mock) (1.0 μg). The luciferase activity of unmethylated pGL3-SNRPN in combination with pPacSp1 and pAc5.1/V5-His (mock) was normalized to 100 (data not shown). Relative luciferase activities (means + standard deviations) are given. The numbers 22, 30, 32, 34, 44, 45, and 52 correspond to R22A, R30A, D32A, Y34A, R44A, S45A, and Y52A, respectively.

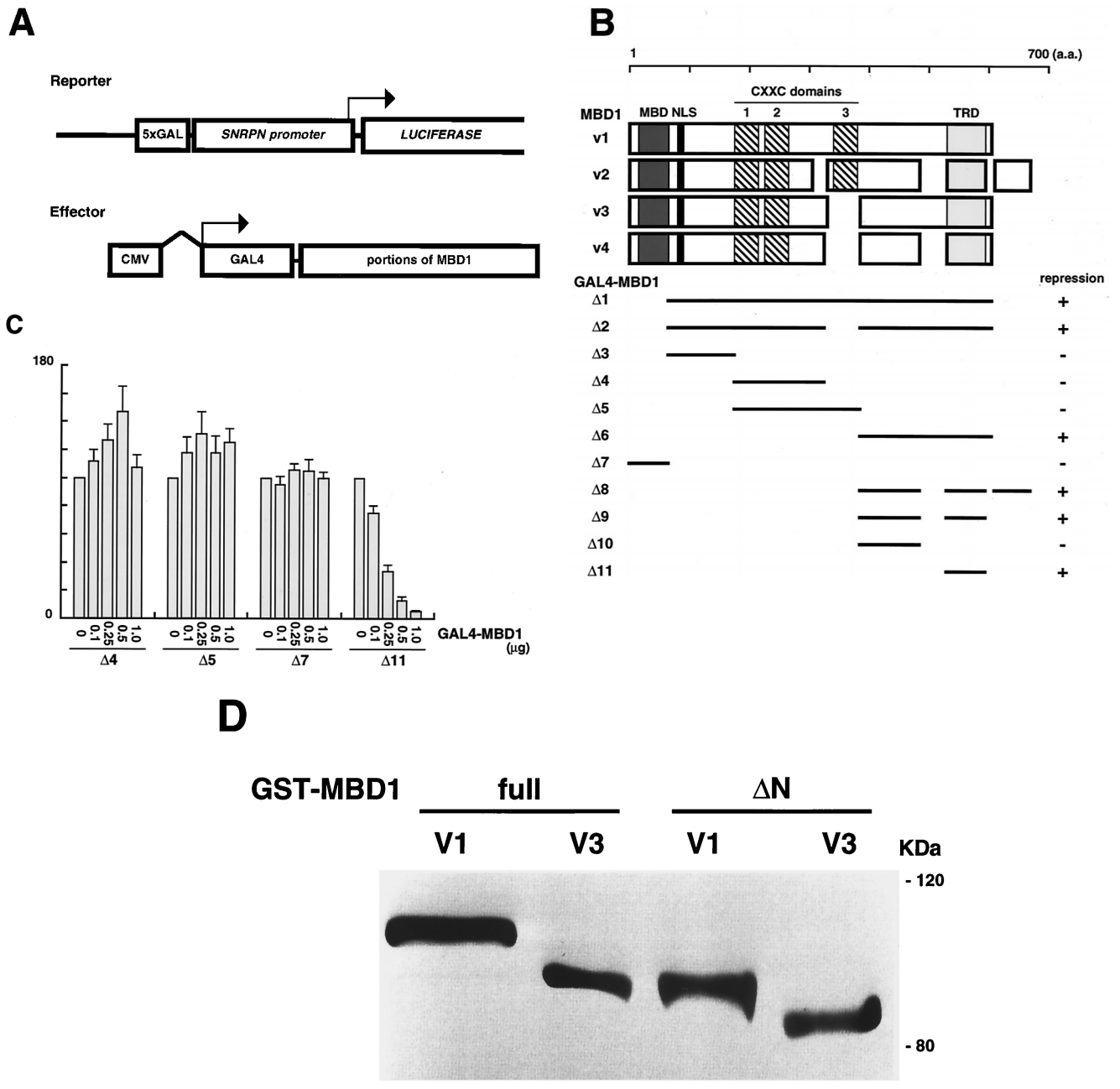

FIG. 6. TRD and DNA binding activity of CXXC3 in MBD1. (A) The reporter construct contains five copies of the GAL4 DNA binding site (5 × GAL) upstream of the *SNRPN* promoter. Effector constructs express the regions of MBD1 fused to the GAL4 DNA binding domain. CMV, cytomegalovirus promoter. (B) Transcriptional repression domain in MBD1 isoforms. Eleven GAL4 fusion proteins (termed GAL4-MBD1Δ1 to GAL4-MBD1Δ11) were constructed: Δ1 (amino acids 62 to 605 of MBD1v1), Δ2 (amino acids 62 to 549 of MBD1v3), Δ3 (amino acids 62 to 173 of MBD1v1), Δ4 (amino acids 62 to 327 of MBD1v3), Δ5 (amino acids 62 to 379 of MBD1v1), Δ6 (amino acids 380 to 605 of MBD1v1), Δ7 (amino acids 1 to 61 of MBD1v1), Δ8 (amino acids 361 to 586 of MBD1v2), Δ9 (amino acids 361 to 523 of MBD1v2), Δ10 (amino acids 361 to 459 of MBD1v2), and Δ11 (amino acids 460 to 523 of MBD1v2). +, shown; −, not shown. (C) Relative transcription levels under the expression of GAL4-MBD1Δ4, GAL4-MBD1Δ5, GAL4-MBD1Δ7, and GAL4-MBD1Δ11. GAL4-MBD1Δ11 specifically repressed transcription from the reporter. (D) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GST-fused MBD1v1 and MBD1v3 stained with Coomassie blue: full-length (full) and with deletion of the MBD (amino acids 1 to 61) (ΔN). (E) Band shift analysis of methylated and unmethylated DNAs complexed with recombinant MBD1. Unmethylated (M−) and *Sss*I-methylated (M+) DNAs of the *SNRPN* promoter were incubated with one of the GST-MBD1 proteins. The CXXC3 of MBD1v1 has a DNA binding activity. Numbers to the right are molecular masses (in kilodaltons).

hemimethylated DNA (3) or methyl-CpG · TpG mismatches that are the primary product of deamination at methyl-CpG (14). Besides, we have shown that recombinant MBD of MBD1 efficiently binds to densely methylated DNAs and that MBD1 localizes to the hypermethylated region of chromosome 1q12 as well as DAPI (4′,6′-diamidino-2-phenylindole)-brightened regions in the nucleus of human cells (10). In contrast, MeCP2 was found to bind preferentially to a single methyl-CpG pair (24) and MeCP2 is distributed throughout the nucleus in human cells (10, 23). Thus, the MBD of these MeCPs

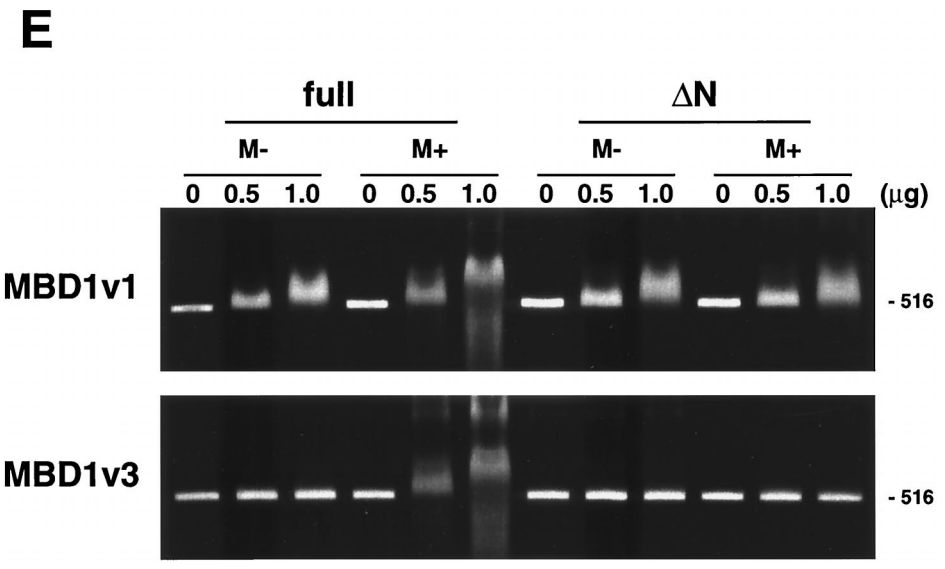

FIG. 6—*Continued*.

appears to be homologous in sequence but distinct in function. Recently, we have reported the structure in solution of the MBD from MBD1 (residues 1 to 75) (29), which is similar to the data from MeCP2 (38). The three-dimensional structure of the MBD of MBD1 shows a compact fold, and assumes an α-β sandwich formation: a four-stranded twisted β-sheet (strand β1, residues 6 to 8; strand β2, residues 15 to 20; strand β3, residues 32 to 37; strand β4, residues 41 to 43), and a helix (α1, residues 47 to 53) with a characteristic hairpin loop at the C terminus. Five residues within the MBD—Arg22, Arg30, Arg44, Tyr34, and Asp32—were suggested to be important for the DNA binding based on the NMR structure and mutagenesis in vitro. In all the other MBD-containing proteins, the positions of these residues correspond to those in MBD1. Mammalian MBD3, however, has histidine and phenylalanine at positions 30 and 34, respectively (13, 29). The fact that MBD1 mutants R30A and Y34A both lose their DNA binding activities in vitro and in the cells supports the weaker selectivity of MBD3 to methylated DNA (37, 41). Further, we have observed that MBD1 is likely to repress transcription by the promoter occupation via MBD-methylated DNA contact. The smallest deletion mutant containing MBD and NLS localizes in the nucleus and moderately inhibits methylated promoter activities (MBD1v1Δ4 in Fig. 3 and wild-type MBD1 in Fig. 5B). In addition, MBD1 mutants S45A and Y52A retained some of their DNA binding activities in vitro and in the cells but could not repress transcription from methylated promoters. Thus, the MBD plays an important role in the methylation-mediated transcriptional silencing. As a matter of some concern, MBD1ΔN (Fig. 2) and some of the MBD1 point mutants (Fig. 5) activated transcription from methylated promoters, probably due to a dominant effect. This suggests that MBD1 interacts with other cellular factors and forms certain complexes via either the CXXC domains or the C terminus. In fact, MeCP2, MBD2, and MBD3 are embedded in the histone deacetylase complexes and are involved in packing the genomic DNA into the inactive chromatin, leading to transcriptional repression (15, 25, 28, 37, 41). Until now, MBD1 has not been found in known histone deacetylase complexes nor in the MeCP1 complex (28). The addition of histone deacetylase inhibitor decreased the relative transcriptional repression of a methylated simian virus 40 promoter by MBD1 with CXXC2 and CXXC3 (27).

We have also focused on the characterization of the CXXC domains and C-terminal TRD in MBD1 isoforms. The data from mammalian and *Drosophila* cells were basically consistent. MBD1v1 repressed transcription from both unmethylated and methylated promoters, while MBD1v3 inhibited methylated but not unmethylated promoter activities. Importantly, MBD1v1 and -v3 preferentially repressed sparsely (*Hpa*II- and *Hha*I-) and densely (*Sss*I-) methylated promoters, respectively. How do MBD1 isoforms repress transcription in a methylation-dependent manner? Are there other mechanisms by which MBD1 can bind and inhibit the gene promoter? The effects of the fragments of MBD1 fused to the DNA binding domain of GAL4 on a reporter gene revealed the presence of the TRD at the C terminus of MBD1. Interestingly, this TRD is perfectly conserved in all the MBD1 isoforms. In addition to recent data collected by Ng et al. (27), our results gave enough evidence that MBD and any of the CXXC domains themselves have no abilities to repress transcription. Thus, MBD1 isoforms inhibit gene expression through both promoter occupation and active TRD function. Finally, the role of the CXXC3 was elucidated by both luciferase and band shift analyses. The CXXC3 sequence in MBD1v1 is exclusively required for the transcriptional silencing of unmethylated and hypomethylated promoters, indicating that each of the CXXC domains has a different role in the transcriptional regulation. From the sequence alignment of the CXXC domains (each 45 residues), the sequence identities for CXXC3 are 33.3, 35.5, 40.0, and 48.9% in the CXXC1, CXXC2, DNA methyltransferase, and ALL-1–HRX, respectively (8). We finally demonstrated that the CXXC3, but not CXXC1 or CXXC2, has DNA binding capacity, regardless of the methylation status. For this reason, MBD1v1 can affect transcription from unmethylated and hypomethylated promoters. However, MBD1 with deletion or point mutations in the MBD failed to repress methylated promoters and reversely augmented transcription, especially from hypermethylated promoters. This observation from repeated experiments may be due to the altered interaction between the

MBD1 mutants, methylated plasmids, and certain endogenous factors within the nucleus.

In conclusion, MBD1 regulates transcription from unmethylated as well as methylated promoters. The MBD of MBD1 binds to methylated DNA and occupies transcription regulatory sequences, resulting in the repressive effect. The CXXC3 of MBD1 can interact with unmethylated as well as methylated DNAs, which is controlled by alternative splicing events. The TRD at the C terminus of the protein actively represses transcription, probably from both unmethylated and methylated promoters.

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